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DOI: https://doi.org/10.1097/MAT.0000000000000255

Intensive Hemodialysis Preserved Cardiac injury

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Cardiac injury triggers cellular responses involving both cardiomyocytes and nonmuscle cells to process cardiac structural remodeling. End-stage renal disease (ESRD), despite conventional dialysis, is associated with adverse cardiac remodeling and increased cardiovascular events. Intensification of hemodialysis with nocturnal home hemodialysis (NHD; five sessions per week; 6–8 hours per treatment) was associated with regression of left ventricular hypertrophy and downregulation of genes in apoptosis and fibrosis. In this pilot study, we hypothesize that NHD achieves its cardiac effects in part through attenuation of innate immune activation resulting in amelioration of cardiomyocytes apoptosis and fibrosis. Eight patients (4M:4F; age, 59 ± 9 years) with ESRD were studied. Half of the cohort was converted to NHD, whereas the rest of the patients were maintained on conventional hemodialysis (CHD). At baseline, CHD was associated with an increase in cardiomyocyte apoptosis detected by flow cytometry using Annexin V (mean fluorescence index in CHD and in normal control is 1.00 ± 0.05 *vs.* 0.66 ± 0.01, $p < 0.05$). After conversion to NHD, cardiomyocyte apoptosis was reduced compared with baseline CHD situation ($p < 0.05$) and approached that of normal control (0.59 ± 0.09 *vs.* 0.66 ± 0.01, $p > 0.05$). The CHD serum was associated with a coordinated augmentation innate immunity pathway, significantly increasing myeloid differentiation factor-88 and interleukin-1 receptor–associated kinase-4; NHD was able to reduce their levels. Heat shock protein 60 was augmented during CHD condition and fell after NHD. In addition, CHD increased fibroblast proliferation and myofibroblast transformation. Uremia is associated with activation of common innate immune signaling pathways leading to fibrosis and apoptosis. Amelioration of uremic clearance by NHD may attenuate this pathological signaling cascade. *ASAIO Journal* 2015; 61:613–619.

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Submitted for consideration August 2014; accepted for publication in revised form April 2015.

The first two authors contributed equally to this work.

Disclosure: The authors have no conflicts of interest to report.

This study was supported in part by the Baxter – Extramural Grant Program. Part of the gene expression array analysis is supported through grants from the Heart & Stroke Foundation and the Canadian Institutes of Health Research.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text, and links to the digital files are provided in the HTML and PDF versions of this article on the journal's Web site (www.asaiojournal.com).

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DOI: 10.1097/MAT.0000000000000255

Key Words: apoptosis, cardiomyocyte, innate immunity, heat shock protein, nocturnal home hemodialysis

All forms of cardiac injury trigger cellular responses involving both cardiomyocytes and nonmuscle cells leading to cardiomyocyte death, cardiac fibroblast proliferation, and fibrosis.[1,2] Cardiomyocyte death has been recently identified as an important novel marker of cardiac pathologies including heart failure, left ventricular hypertrophy (LVH), myocardial infarction, and ischemia/reperfusion injury.[3,4] Indeed, the extent of cardiomyocyte damage was shown to be an independent predictor of clinical outcomes in patients with dilated cardiomyopathy.[5]

Cardiovascular disease continues to be the leading cause of death in patients with end-stage renal disease (ESRD) undergoing conventional hemodialysis (CHD) three times a week. Intensive home nocturnal home hemodialysis (NHD; 5–6 times per week, 6–8 hours per session) has been shown to be associated with regression of LVH[6] and other cardiovascular surrogate outcomes.[7–10] Recently, our team reported that intensification of hemodialysis dose was also associated with a coordinated downregulation of genes that are responsible for apoptosis and fibrosis (cyclin-dependent kinase inhibitor 1A [Cdkn1a] and cyclin-dependent kinase inhibitor 1C [Cdkn1c]), Fas, and Bcl2-associated X protein (Bax).[11] In this pilot study, we aim to examine the pathway by which NHD may attenuate the process of cardiac injury.

We hypothesize that soluble substances in the uremic plasma from CHD patients exposed to cardiomyocytes will trigger an innate immune response resulting in apoptosis of cardiomyocytes, and conversion from CHD to NHD will decrease the extent of innate immunity–mediated cardiomyocytes apoptosis. In contrast, patients maintained on CHD will be associated with similar degree of cardiomyocyte apoptosis. The present exploratory study will form the basis for a comparative trial.

Methods

This protocol was approved by the Research Ethics Board of the Toronto General Hospital, University Health Network, Toronto, Canada, and conformed to the standards established by the Declaration of Helsinki. Medically stable ESRD patients (age between 18–85 years) who have received a minimum of 3 months of CHD were invited to participate in this study. None of the patients had any acute illness, hospitalization, or symptomatic cardiovascular disease (including congestive heart failure and acute coronary syndrome). Normal subjects were also recruited. Written informed consent was obtained from each patient. Pregnant patients were excluded. Herein, we will describe the clinical aspects of the protocol, followed by the *in vitro* experimental details.

Subjects

Subjects included consecutive eligible patients who were maintained on CHD or being converted from CHD to NHD at

Table 1. Demographic and Clinical Characteristics

Variable	ESRD
N	8
Age (yr)	59 ± 9
Gender (M:F)	4:4
Etiology of ESRD	
Glomerular disease	1
HTN	2
Type 2 diabetes	3
PCKD	1
Systemic lupus erythematosus	1
Diabetes (%)	50
Antihypertensive medications (%)	
ACEI or ARB	63
Calcium channel blocker	40
β-Blocker	63
Vascular access	
Arteriovenous fistula	4
Central venous catheter	4

ACEI, angiotensin-converting enzyme inhibitors; ARB, angiotensin receptor blockers; ESRD, end-stage renal disease; F, female; HTN, hypertension; M, male; PCKD, polycystic kidney disease.

the University Health Network. Patient demographic information, such as age, gender, ethnicity, etiology of ESRD, and comorbid conditions, was prospectively collected into a computerized clinical database. Medications were recorded. Clinical assessment, including weight, height, and blood pressure measurements, was performed at baseline and monthly after conversion to NHD. All blood pressure measurements were made at the patients' home using a calibrated blood pressure cuff after 5 minutes of rest at the sitting position. Biochemical and hematological parameters (complete blood count, urea, creatinine, albumin, calcium, phosphate, and parathyroid hormone) were obtained monthly during the same time intervals. Baseline and subsequent 1 year blood samples were collected predialysis at the same time of day. In the case of NHD, a minimum of 4 hours after the regular NHD session was ensured to approximate steady-state condition. Eight patients (4M:4F; age, 59 ± 9 years) with ESRD were studied. Description of our study patient cohort is outlined in **Table 1**.

Dialysis Protocol

CHD patients received hemodialysis for 4 hours 3 times per week by similar vascular access. A blood flow rate of 400 ml per minute, a dialysate flow rate of 500–750 ml per minute, and F80 polysulfone dialyzers (Fresenius Medical Care, Lexington, MA) were used. The NHD patients received hemodialysis at home for 6–8 hours, 5–6 nights per week. Vascular access was achieved through either a long-term internal jugular catheter (Uldall Catheter, Cook Critical Care, Bloomington, IN) or an arteriovenous fistula. Dialysate flow rate of 350 ml per minute and blood flow rate of 200–300 ml per minute was used. F80 polysulfone dialyzers (Fresenius Medical Care) or Xenium dialyzers (Baxter, Chicago, IL) were used. Unfractionated heparin was used for anticoagulation on CHD and NHD.

Dialysis dose per treatment was estimated by equilibrated Kt/V (eKt/V) as described by Daugirdas *et al*.[12]: eKt/V = spKt/V − 0.6(spKt/V)/t + 0.03, where spKt/V = single-pool Kt/V, K = delivered clearance, t = dialysis time, and V = urea distribution volume. Single-pool Kt/V was determined using blood urea reduction ratio.[13]

Neonatal Cell Culture Preparation

Ventricular myocytes and cardiac fibroblast were isolated from 2-day-old neonatal C57/B6 mice. In brief, neonatal mouse myocytes were isolated and cultured as described previously.[14,15] A single litter of 1- to 2-day-old mice was used for each experiment. Pups were killed by cervical dislocation, and the hearts were removed quickly into filter-sterilized buffer. By using aseptic technique, atria and blood vessels were removed, and the ventricles were minced. Ventricular tissue was dissociated at room temperature (22–24°C) with trypsin and gentle mechanical agitation. Cells were collected in fetal calf serum (FCS, Gibco, Grand Island, NY). When dissociation was complete, the cell suspension was centrifuged, washed once, and resuspended in culture medium (Dulbecco's Modified Eagle Medium: HAM F-12 [Gibco], 1:1, 10% v/v FCS; penicillin/streptomycin, 50 unit/ml). After preplating, cardiomyocytes were collected and plated in the laminin-coated 6-well culture dishes (Nunc, Thermo Scientific, USA) at a density of 3×10^5 viable cells/ml (as determined by trypan blue staining) in culture medium supplemented with 5-bromo-2-deoxyuridine (0.1 mM; Sigma-Aldrich Co LLC). Cells were incubated at 37°C in a humidified atmosphere of 1.5% CO_2. After 24 hours in culture, the medium was replaced by serum-free medium, and 24 hours after serum starvation, each cell groups were treated with 10% human plasma (under CHD, NHD, or normal conditions) or with fetal bovine serum as a control.

Apoptosis and Fibroblasts Proliferation Assessment Using Flow Cytometry

To quantify apoptosis, cells were washed with cold 1x phosphate-buffered saline twice and resuspended in 1x Annexin V

Table 2. Changes in Biochemical Parameters

	Control Group Baseline	Control Group Final	NHD Group baseline	NHD Group Final
Variables (N)	4	4	4	4
Albumin (g/L)	36.2 ± 1.9	36.0 ± 1.9	36.2 ± 2.2	37.5 ± 1.3
Phosphate (mmol/L)	1.44 ± 0.4	1.67 ± 0.1	1.49 ± 0.43	1.38 ± 0.15
PTH (nmol/L)	16.0 ± 7.2	38.6 ± 18.8	27.9 ± 13.0	31.6 ± 10.8
Systolic blood pressure (mmHg)	139 ± 7	140 ± 16	139 ± 9	145 ± 10
Diastolic blood pressure (mmHg)	72 ± 7	73 ± 7	70 ± 4	69 ± 3
Sodium (mmol/L)	135 ± 2	138 ± 1	137 ± 2	135 ± 2
Number of antihypertensive medications	2 ± 1	2 ± 1	2 ± 1.4	1 ± 1

NHD, nocturnal home hemodialysis; PTH, parathyroid hormone.

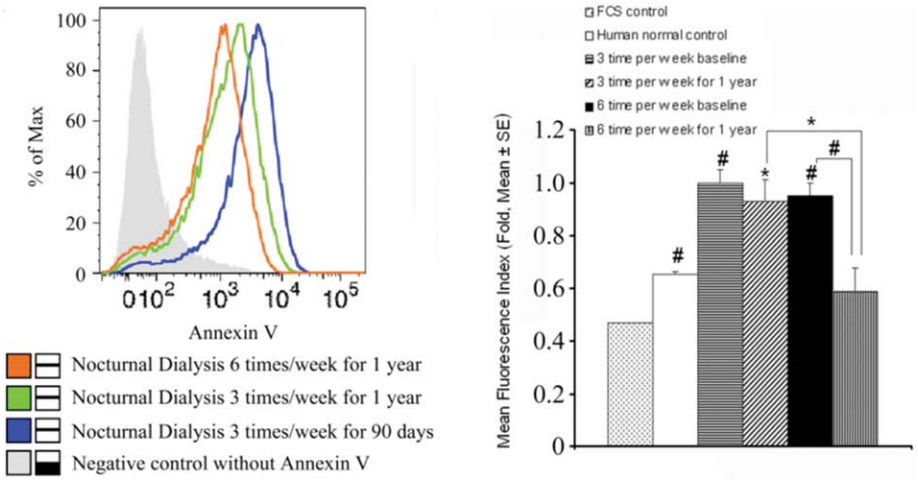

Figure 1. Neonate cardiomyocyte apoptosis detected by flow cytometry using Anexin V in different groups. Left: A representative figure of flow cytometry. Right: Quantification by mean fluorescence index. *$p < 0.05$; #$p < 0.01$. FCS, fetal calf serum; SE, standard error.

binding buffer. Staining with fluorescein isothiocyanate–conjugated Annexin V was performed according to commercial instruction (Biolegend, San Diego, CA). To evaluate the proliferation of cardiac fibroblast, we plated cardiac fibroblast cells in 3×10^5/well in 6 well plates. Cells were allowed to grow overnight and were treated with 0.1 μM carboxyfluorescein diacetate succinimidyl ester (CFSE, CellTrace CFSE Cell Proliferation Kit, Molecular Probes) for 30 minutes at 37°C in 5% carbon dioxide. Cells were washed with five volumes of medium for three times. The cell preparation were treated by different groups of patients' plasma for 24 hours and then harvested. Carboxyfluorescein diacetate succinimidyl ester diffused passively into cells. Fibroblasts were labeled through the reaction of succinimidyl ester group and intracellular amines, forming fluorescent conjugates and analyzed using a flow cytometer.

Reverse Transcription and Real-Time Quantitative PCR

Total RNA was isolated from neonate cardiomyocytes as described earlier after treatment with 10% human plasma (under CHD and NHD conditions; n = 3 for each group). Reverse transcription was performed using Superscript II (Invitrogen, CA) according to the manufacturer's instruction. Gene expression levels were quantified by real-time polymerase chain reaction (PCR). All reactions were run in triplicates. The primers used for this study are shown in Supplemental Table 1 (see Supplemental Digital Content 1, http://links.lww.com/ASAIO/A66). The ratio of the level of each gene measured to GAPDH was estimated with the ΔCT ($Ct_{Taget} - Ct_{GAPDH}$) method. The relative gene expression (fold change with respect to baseline) was estimated with the 2-ΔΔCT method.[16]

Immunoblotting

Protein levels were examined by immunoblotting as described previously.[16] Primary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX; GAPDH, α-smooth muscle [SM] actin), Cell Signaling (Danvers, MA; myeloid differentiation factor-88 [MyD88] and interleukin-1 receptor–associated kinase-4 [IRAK-4], heat shock protein [Hsp]: Hsp27, Hsp60, Hsp70, and Hsp90), Stressgen (San Diego, CA; tumor necrosis factor receptor–associated factor 6 [Traf6], and R & D Systems Inc. (Minneapolis, MN; fibroblast growth factor [FGF] 23). Equal amounts of protein (20–50 μg) were loaded in each lane of 4–20% Tris-glycine gel (Novex, Invitrogen, CA). The bands were analyzed by using the Quantity One software (Hercules, CA) and normalized by GAPDH. The results from each group of experiment were described as the number of fold changes relative to baseline levels.

Data Analysis

Descriptive data are presented as mean ± SE unless otherwise indicated. The primary outcome measure was the within-subject paired difference in gene expression before and after conversion from CHD to NHD. Secondary outcomes included between-subject differences in gene expression. Differentially expressed genes were identified as described before and after conversion to NHD. Mann-Whitney *U* test was used for comparison of continuous variables between two groups. Analysis of variance was used for multiple comparisons of a continuous variable among four groups of subjects. Spearman's correlation was used to investigate potential associations between variables of interest. All statistical tests were two tailed with a *p* value less than 0.05 taken to indicate significance. SPSS version 10 (SPSS Inc., Chicago, IL) was used for all statistical analyses.

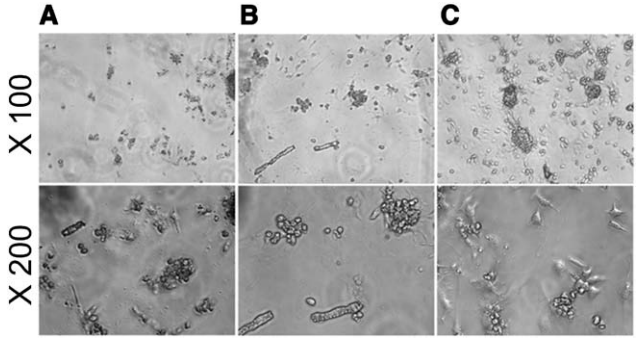

Figure 2. Photomicrograph of cardiomyocytes in culture. **A**: Conventional hemodialysis condition (baseline). **B**: Conventional hemodialysis condition (1 year). **C**: Nocturnal hemodialysis condition.

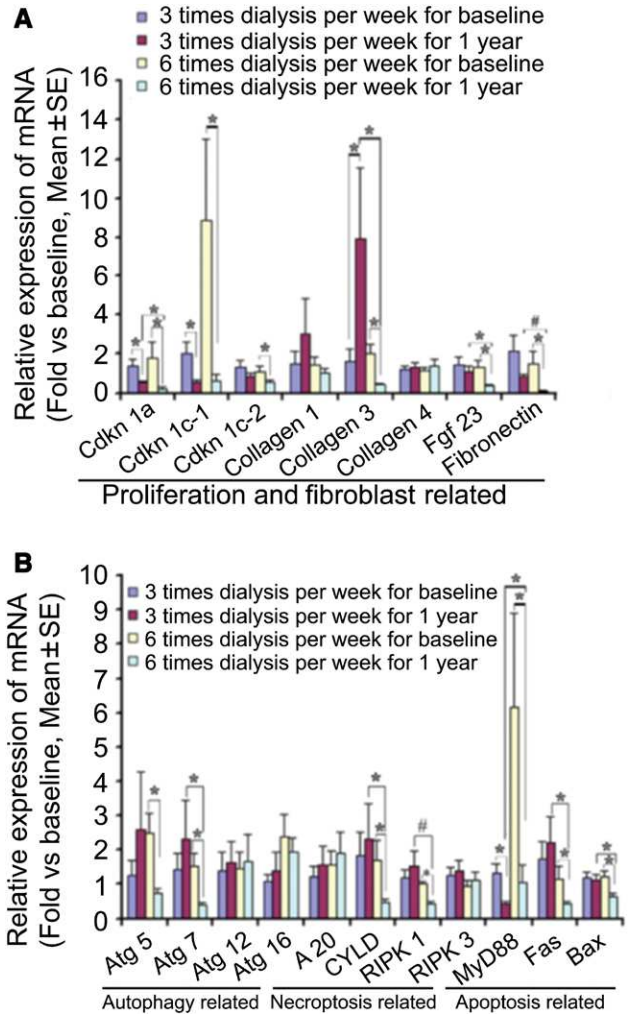

Figure 3. Gene expression patterns under different hemodialysis conditions. Cdkn1a, cyclin-dependent kinase inhibitor 1A; Cdkn1c, cyclin-dependent kinase inhibitor 1C; Fgf23, fibroblast growth factor 23; Atg, autophagy; CYLD, cylindromatosis; RIPK, receptor-interacting protein kinase; Bax, Bcl2-associated X protein. *p < 0.05; #p < 0.01. SE, standard error.

Results

Eight patients (4M:4F; age, 59 ± 9 years) with ESRD were studied. Their comorbid conditions were listed in **Table 1**. Ninety percent of our study population had a diagnosis of hypertension and was prescribed antihypertensive medications. Half of the cohort was converted to NHD, whereas the rest of the patients were maintained on CHD. After conversion to NHD, sessional Kt/V increased from 1.20 ± 0.2 to 2.20 ± 0.2, $p < 0.05$. Standardized weekly Kt/V increased from 2.20 ± 0.2 to 5.50 ± 0.5, $p < 0.05$ in the NHD cohort, whereas it remained unchanged in the CHD patients. There was no between-group differences in either baseline or follow-up values in blood pressure, hemoglobin, phosphate, or albumin (**Table 2**).

NHD Was Associated with Reductions in Cardiomyocyte Apoptosis and Fibroblast

At baseline, CHD was associated with an increase in cardiomyocyte apoptosis detected by flow cytometry using Anexin V (**Figure 1**). Mean fluorescence index in CHD and in normal control was 1.00 ± 0.05 *vs*. 0.66 ± 0.01, respectively, $p = 0.01$). After conversion to NHD for 1 year, cardiomyocyte apoptosis (0.59 ± 0.09) was reduced compared with baseline CHD condition ($p = 0.02$ and $p < 0.05$) and approached that of normal control ($p = 0.69$). In contrast, cardiomyocyte apoptosis did not change at baseline and after 1 year of CHD. Qualitatively, neonate cardiomyocytes treated with plasma from patients with CHD (at baseline and after 1 year) showed obvious cell death with myocytes elongation, whereas myocytes treated by plasma from patients with NHD were morphologically intact (**Figure 2, A–C**).

The interactions between cardiac fibroblasts and cardiomyocytes are essential for the progression of cardiac remodeling. We detected that CHD is characterized by a proliferation of fibroblast at baseline (44.1%) and at 1 year (29.1%). In contrast, NHD reduced the proportion of proliferating fibroblasts from 45.4% (baseline) to 15.5% (NHD for 1 year). In addition, fibroblasts phenotype changed to myofibroblast with increased expression of α-SM actin (data not shown). Of note, collagen III production was stimulated during CHD conditions, which was subsequently attenuated after conversion to NHD (**Figure 3**).

NHD-Induced Gene Expression Changes in Cardiomyocytes

A priori, we targeted genes associated with fibroblast proliferation and cardiomyocyte autophagy, necrosis, and apoptosis (**Figure 3**). Nocturnal home hemodialysis was associated with downregulation in Cdkn1c-2, Cdkn1a, FGF23, collagen 3, and fibronectin, implicating that NHD is less fibrogenic compared with CHD. Intensification of dialysis dose did not modify consistently all genes associated with autophagy. In contrast, NHD is associated with downregulation of MyD88, Fas, and Bax.

Uremia-Induced Cardiomyocyte Apoptosis Through Activation of the Innate Immunity Pathway

We systematically analyzed the influence of conversion from CHD to NHD on MyD88 mediated pathway (**Figure 4**). At baseline, Traf6 and IRAK-4 were increased under CHD condition. After 1 year of NHD, MyD88, Traf6, and IRAK-4 were reduced, whereas CHD did not modify the levels of these intermediate activation nodes. Given that Hsps) are known to trigger MyD88 pathway, systematically, we examined Hsp27, Hsp60, Hsp70, and Hsp90 levels at baseline and after 1 year of CHD or NHD. Of note, we observed that Hsp60 was increased at baseline and was reduced after conversion to NHD (**Figure 5**).

We also examined the levels of RIPK1 and RIPK3, two important nodes in the necroptosis pathway. Changes in dialysis prescription did not alter the necroptosis pathway (Data not shown).

Discussion

Cardiovascular disease remains the leading cause of death in patients with ESRD. We have previously reported the gene signature of neonatal cardiomyocytes exposed to uremic serum before and after conversion from CHD to NHD. The present results extend our observations by which uremia

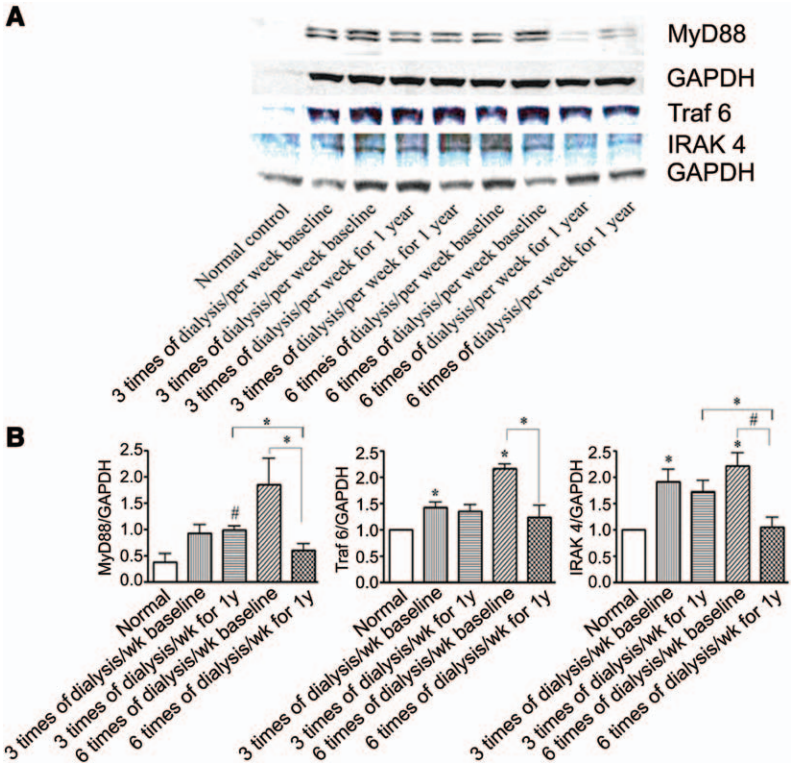

Figure 4. Innate immunity activation under different hemodialysis conditions. **A**: Representative Western blotting analysis is shown. **B**: Quantitative analysis is shown. MyD88, myeloid differentiation factor-88;, Traf6, tumor necrosis factor receptor–associated factor 6); IRAK-4, interleukin-1 receptor–associated kinase-4. full color online

is associated with cardiomyocyte apoptosis. Of the potential candidate pathways, our data suggest that uremia is associated with activation of the ubiquitous toll-like receptors (TLR) and their downstream signaling pathways such as MyD88 and IRAK-4 leading to activation of innate immunity and apoptosis.

Inflammatory processes are fundamental to common cardiovascular diseases such as myocardial infarction, heart failure, and atherosclerosis. Of the various inflammatory response models, the "Danger Model" proposes that the immune system response leads to harmful signals that cause tissue stress and ultimately destruction.[17] Differences in the type of stress will determine the severity and extent of immunological response. Tissue stresses may include classical bacterial/viral infection, ischemia/reperfusion/oxygen-free radicals, mechanical injury, and transplantation. Classically, triggers to "danger signals" would include lipopolysaccharide and endogenously produced Hsp. The innate immune response to Danger Signals include the TLRs signaling pathways and their downstream signaling pathways such as MyD88 or IRAK-4 leading to ultimately activation of transcription factors such as nuclear factor (NF)-κB and in turn mobilization of natural killer or dendritic cells and activation of acquired immunity.[18]

Recently, the cardiac relevance of the Danger Signal hypothesis was substantiated by Kim *et al*.[19] These investigators treated adult rat cardiomyocytes with Hsp60. They demonstrated that Hsp60-induced apoptosis of cardiomyocytes (as measured by increased caspase 3 activity and increased DNA fragmentation). In addition, apoptosis could be reduced by blocking antibodies to TLR4 and by NF-κB binding decoy. Clinically, circulating Hsp60 has been detected in plasma of patients with heart failure[20] and represents a logical cardiac biomarker in high-risk population. Emerging data also support the importance of innate immunity in the pathogenesis of LVH. Toll-like receptor 4 antagonist (eritoran) was applied in a murine model after transverse aortic constriction. In comparison with the placebo arm, eritoran-treated animals had lower levels of interleukin-1 and interleukin-6.[21] Left ventricular mass was also reduced in comparison with the placebo arm. Similarly, MyD88 inhibitor was administered in murine model of acute myocardial infarction. MyD88 inhibition attenuated left ventricular dilatation and LVH, suggesting that Danger Signal Innate Immune Amplification System may be a viable target for treatment of LVH.[22] In addition, the role of innate immunity has also emerged as a modulator of blood pressure and vascular injury.[23] It is interesting to note that intensive hemodialysis has been consistently shown to improve blood pressure control, and it is tempting to speculate that the present results may be in part because of the improved blood pressure regulation.

Although both LVH and inflammation are known to contribute to cardiovascular events in ESRD, it is unclear whether uremia *per se* may directly induce an inflammatory response. Toll-like receptor expression has also been studied in patients with ESRD. Gollapudi *et al*.[24] examined blood samples from 21 stable hemodialysis patients and 21 normal controls. The ESRD group exhibited significant upregulation of TLR2 and 4 expressions on monocytes and significant upregulation of TLR4 on polymorphonuclear leukocytes. In addition, TLR4 activity (as measured by lipopolysaccharide-stimulated cytokine production) was higher in hemodialysis patients. Interestingly, in a separate study, TLR4 expression in peripheral

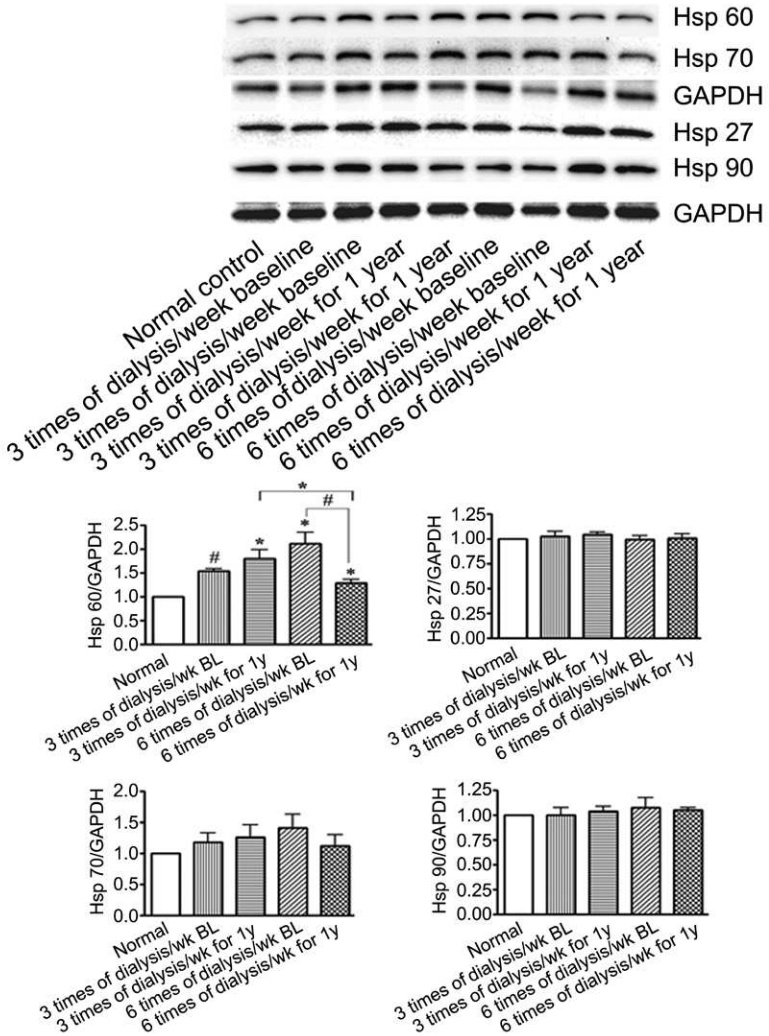

Figure 5. Differences in Hsp expression under different hemodialysis conditions. **A**: Representative Western blotting analysis is shown. **B**: Quantitative analysis is shown. BL, baseline; Hsp, heat shock protein.

blood monocytes was evaluated in 191 patients with ESRD.[25] TLR4 expression correlated significantly with age ($r = 0.2$, $p = 0.007$), CRP ($r = 0.2$, $p = 0.008$), and mean arterial blood pressure ($r = -0.2$, $p = 0.02$).

Along with cardiomyocyte death, fibrosis aggravates the extent of cardiac injury. External stress causes fibroblasts to change their phenotype,[26,27] referred to as myofibroblasts because they express several SM markers including α-SM actin and SM22α.[27–29] Myofibroblasts appear in the myocardium and believed to arise from resident interstitial and/or adventitial fibroblasts after injury[26] but are not found in normal cardiac tissue.[30] The disproportionate increase in collagen synthesis and deposition lead to stiffening of ventricles and impede both systolic and diastolic functions.[31] In addition, fibrosis reduces capillary density and increases oxygen diffusion distance leading to hypoxia of cardiomyocytes, which is well recognized in ESRD.[32,33] Our group has previously reported amelioration in myocardial mechanics and ventricular stiffness before and after conversion from CHD to NHD.[12]

The present results will form the basis for a more robust examination of the notion that intensive hemodialysis may attenuate cardiac injury by decreasing the extent of cardiomyocyte apoptosis and innate immunity activation. We will aim to use plasma collected from patients randomized to the Frequent Hemodialysis Network. Plasma from these patients will then be used to culture cardiomyocytes using the aforementioned techniques. We will aim to ascertain whether the degree of cardiomyocyte apoptosis and innate immunity activation is associated with the extent of left ventricular mass regression.

In summary, our pilot study is associated with a novel pathway by which uremia may cause cardiac damage by immune amplification. Our results suggest that uremia clearance directly impacts on cardiomyocyte apoptosis and myofibroblast transformation. The clinical relevance of innate immunity in patients with ESRD will be tested in a more robust manner using samples stored from randomized controlled trials.

Acknowledgment

Dr. Christopher Chan holds the R Fraser Elliott Chair in Home Dialysis. Dr. Peter Liu is the Heart & Stroke/Polo Chair Professor of Medicine and Physiology at the University of Toronto.

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